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Alendronate and Etidronate do not Regulate Interleukin 6 and 11 Synthesis in Normal Human Osteoblasts in Culture

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Abstract. Bisphosphonates exert a potent inhibitory effect on bone resorption. Several studies have been performed, with contradictory results, to ascertain whether the effect of bisphosphonates on osteoclasts could be produced, at least in part, by modulation of the synthesis of resorption-promoting factors by osteoblasts. The aim of this study was to evaluate the effect of etidronate (10^{-4}–10^{-9} M) and alendronate (10^{-7}–10^{-12} M) on the production of IL-6 and IL-11 using human osteoblast cultures. Cytokines were quantified by ELISA, and mRNA expression was tested. Treatment with alendronate and etidronate had no effect on the synthesis of IL-6 or IL-11, and IL-6 and IL-11 mRNA levels. These results were obtained both in nonstimulated cultures and in cultures stimulated by means of TNF-α, IL-1β, and TNF-α + IL-1β, with or without FCS. In conclusion, a possible indirect osteoclast-mediated effect of alendronate and etidronate on bone resorption would not be exerted through reduction in osteoblastic synthesis of IL-6 and IL-11.

Key words: Alendronate — Etidronate — Human osteoblasts — Interleukin 6 — Interleukin 11

Bisphosphonates are potent inhibitors of bone resorption when tested in different systems, both *in vivo* and *in vitro*. At present, there are two models that would explain how this inhibition is produced. On the one hand, owing to the high affinity of bisphosphonates for hydroxyapatite, the inhibiting effect of these bone matrix-linked compounds would result from their direct action on the resorptive ability of osteoclasts [1–3]. Thanks to their endocytotic capacity osteoclasts would internalize bisphosphonates, which in turn would inhibit the metabolic reactions involved in osteoclast function. Bisphosphonates that closely resemble pyrophosphate (such as clodronate and etidronate) can be metabolically incorporated into nonhydrolyzable analogs of ATP. It is likely that intracellular accumulation of these metabolites inhibits osteoclast function. The more potent, nitrogen-containing bisphosphonates (such as pamidronate and ibandronate) are not metabolized but can inhibit enzymes of the mevalonate pathway, thereby preventing the biosynthesis of isopropenoid compounds that are essential for posttranslational modification of small GTPases. These GTPases are important signaling proteins regulating a variety of cell processes required to maintain osteoclast activity and survival [4]. Loss of osteoclast function and apoptosis is probably the consequence of function loss of one or more of these signaling proteins [5–8].

On the other hand, several *in vitro* studies [9, 10] failed to show the differences in inhibitory power on bone resorption found *in vivo* among the different types of bisphosphonates. These differences appeared when osteoclasts were co-cultured with osteoblasts [11]. This finding suggests that the effect of bisphosphonates on osteoclasts could be produced, at least in part, by modulation of the synthesis of resorption-promoting [12] or resorption-inhibiting factors by osteoblasts [13].

Among these resorption-promoting factors, IL-6 has been postulated to be an autocrine/paracrine regulator of bone resorption, and plays a role in the early stages of osteoclastogenesis by promoting the formation of osteoclast precursors [14]. It has also been shown that cells in giant cell tumor of bone (human osteoclastoma) have receptors for this cytokine, which would stimulate the resorptive activity of these cells. These latter findings appear to suggest that IL-6 could influence not only the early stages of osteoclast development, but also the activity of mature osteoclasts [15]. IL-6 is produced, in nanomolar quantities, by different types of cells, including monocyte-macrophages, fibroblasts, and osteoblastic cells [16, 17]. Different factors regulate the production of IL-6 and include parathyroid hormone (PTH), PTH-related protein (PTH-rP), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), estrogens, transforming growth factor (TGF), interleukin-1 (IL-1), and tumor-necrosis factor (TNF) [18]. Also, a recent study in

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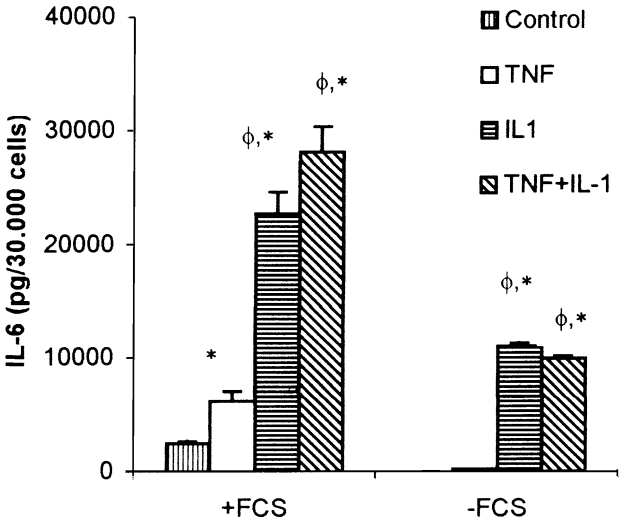

Fig. 1. Effect of TNF-α (0.3 nm) and IL-1β (0.2 nm) on IL-6 induction in human osteoblasts. Cultures were treated for 24 h in the presence or absence of TNF-α, IL-1β, or TNF-α + IL-1β, with or without FCS. The conditioned media were assessed for IL-6 production by human IL-6-specific ELISA. IL-6 synthesis in cultures treated with TNF-α, IL-1β, or TNF-α + IL-1β showed a statistically significant increase compared with those not stimulated (*$P \leq 0.0005$). There are also differences when cultures stimulated with IL-1β or TNF-α + IL-1β are compared with TNF-α cultures (*$P \leq 0.0005$), with or without FCS. Data are expressed as mean ± SEM (n = 3)

cultures of rat osteoblastic cells showed that IL-6 induces its own synthesis in osteoblasts using transcriptional mechanisms [19]. In a preliminary study, Passeri et al. [12] assessed IL-6 production by human osteosarcoma cell-line MG-63, in response to different doses of alendronate and etidronate, and observed a dose-dependent inhibition of IL-6 production by these cells. These data appeared to suggest that IL-6 might be one of the resorption-promoting factors by which bisphosphonates could inhibit the action of osteoclasts.

IL-11 is another cytokine with pleiotropic action whose spectrum of biological activity overlaps that of IL-6. Like IL-6, it stimulates acute-phase protein synthesis [20], B-cell immunoglobulin production [21], and hemopoiesis [22]. IL-11 induces osteoclastogenesis and is associated with osteolysis [23] by mediating the osteoclastogenic effects of PTH, IL-1β, and TNF-α [24]. Osteoblasts and osteosarcoma cells (SaOS-2) have been shown to be potent producers of IL-11 [25]. Even though the action of IL-11 on osteoclasts is similar to the action of IL-6, we are not aware of any report analyzing the effect of bisphosphonates on the production of this cytokine by osteoblasts.

The purpose of the present study was to evaluate the production of IL-6 and IL-11 by normal human osteoblasts *in vitro*, in response to different doses of alendronate and etidronate.

Materials and Methods

Reagents

Dulbecco's phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY). Fetal calf serum (FCS) and trypsin were obtained from Biological Industries (Kibbutz Beth Haemek, Israel). Cedaceria Industrial (Barcelona, Spain) provided nylon mesh, and culture plasticware was purchased from Corning (Corning, NY). $1,25(OH)_2D_3$ was provided by Roche (Basel, Switzerland). Recombinant human IL-1β and TNF-α were obtained from R&D Systems, Inc. (Minneapolis, MN). Alendronate (AHuBP) was generously provided by Dr. Corral at Merck, Sharp & Dohme (West Point, PA); and etidronate (HEBP) was acquired from Laboratorios Rubio (Barcelona, Spain). The IL-6 ELISA kit was purchased from Endogen, Inc. (Cambridge, MA) and the IL-11 kit from R&D Systems, Inc. (Minneapolis, MN). Nylon membranes were from Bio-Rad Laboratories (San Jose, CA).

Normal Human Osteoblast Culture

Cells were cultured as previously described [2, 6]. Briefly, human bone cells were obtained from the surgical specimens of three patients, two women aged 69 and 74 and one man aged 68, all undergoing surgery for degenerative joint disease. Osteoblasts were obtained from 1 to 2 mm explants of trabecular bone placed on an 80 μm mesh maintained between two glass rings and cultured in supplemented medium with 20% fetal calf serum. Approximately 1 week later, osteoblasts started to grow out from the explants over the mesh. Meshes were completely covered by osteoblasts after 3–4 weeks. Cells were isolated using 0.25% trypsin and then subcultured. Osteoblasts were characterized by alkaline phosphatase activity (ALP) and osteocalcin synthesis in response to stimulation with $1,25(OH)_2D_3$. In all cases, cells were stained intensely by a histochemical reaction for ALP and were found to secrete osteocalcin after stimulation with $1,25(OH)_2D_3$. All proliferation and differentiation assays were performed with cells from the third to the fifth passage.

Bisphosphonate Treatment

Alendronate (AHuBP) and etidronate (HEBP) were dissolved in NaCl 0.9% at pH 7.4 to prepare the stock solutions at a concentration of 10^{-3} M and 10^{-2} M, respectively. From these stock solutions, the remaining concentrations were prepared by serial dilutions in DMEM, with or without fetal calf serum (FCS), and added to the osteoblast cultures in concentrations ranging from 10^{-7} to 10^{-12} M for AHuBP and 10^{-4} to 10^{-9} M for HEBP. These concentrations were detected taking into account that the 50 effective dose (ED_{50}) of alendronate as a bone resorption inhibitor is 2×10^{-9} mol/L and that ED_{50} of etidronate as a bone inhibitor is 1×10^{-6} mol/L [11]. The maximum concentration of both bisphosphonates used in the study were chosen since higher concentrations affect osteoblast viability [31]. Each experiment was performed in triplicate.

IL-6 and IL-11 Assays

Cells from the third to the fifth passage were plated in 24-well plates at a density of 3×10^4 cells/well and incubated in DMEM supplemented with FCS. After 24 hours, the medium was changed and the cells were incubated for a further 48 hours with serum-free medium supplemented with 0.1% bovine serum albumin (BSA). The AHuBP, HEBP, or vehicle was then added and the cells were incubated for an additional 24 hours, with or without FCS. Human recombinant IL-1β (0.2 nm) or recombinant murine TNF-α (0.3 nm), or the combi-

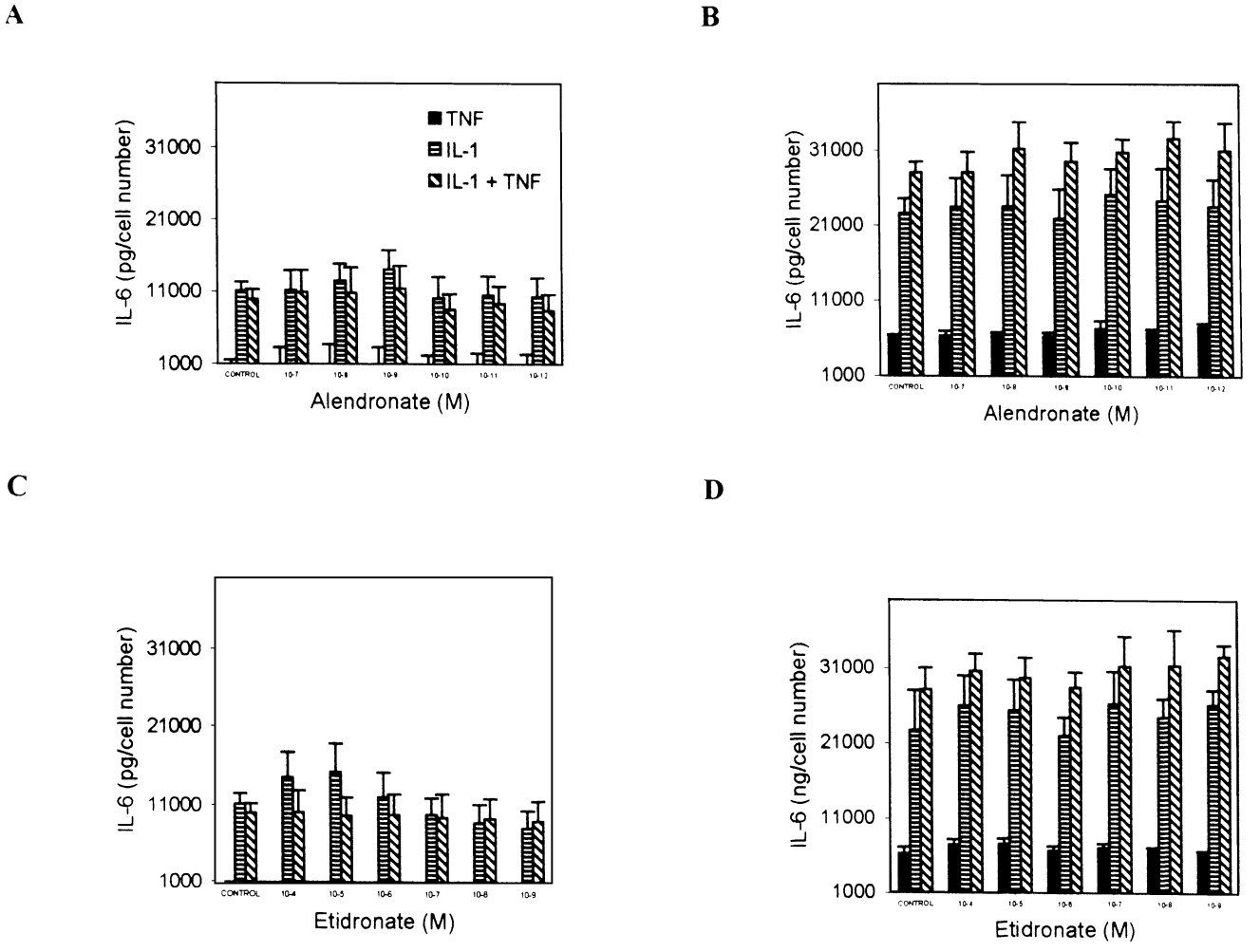

Fig. 2. Effect of different doses of alendronate and etidronate on TNF-α, IL-1β, or TNF-α + IL-1β-induced IL-6 secretion in human osteoblasts. Cultures were treated for 24 h with alendronate or etidronate in the presence or absence of TNF-α, IL-1β, or TNF-α + IL-1β, with or without FCS. **(A)** Cultures treated with alendronate without FCS, **(B)** alendronate with FCS, **(C)** etidronate without FCS, **(D)** etidronate with FCS. The conditioned media were assessed for IL-6 production by a human IL-6-specific ELISA. No effect of any of the alendronate or etidronate concentrations tested was observed on IL-6 synthesis in the cultures, with or without TNF-α, IL-1β, or TNF-α + IL-1β, stimulation, with or without FCS. Data are expressed as mean ± SEM (n = 3).

nation of IL-1β + TNF-α, were also added. At this stage, the supernatants were collected and frozen at −80°C. IL-6 and IL-11 were measured in the conditioned medium by an enzyme-linked immunoabsorbent assay (ELISA). The IL-6 kit detection limit was 1 pg/ml, and that of IL-11 was 8 pg/ml, IL-6 and IL-11 were determined in medium with or without FCS to rule out their presence in the medium or serum which might alter the results of the study.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was purified using the single-step acidified guanidinium thyocianate phenol-chloroform extraction method [27]. Equal amounts of RNA were size-fractioned by electrophoresis on 1% agarose formaldehyde gels and transferred by passive blotting to nylon membranes. To confirm equal loading and the integrity of RNA samples, the gels were stained with ethidium bromide and ribosomal RNAs (28s and 18s) visualized by UV transillumination. The RNA blots were prehybridized, hybridized with ^{32}P-labeled cDNA probes, washed under conditions of high stringency, then studied by autoradiography. Clone pHuIL-11/PMT, a 1,250 base pair IL-11 cDNA in the EcoRI site of the PMX vector, was kindly provided by Dr. Paul Schendel (Genetics Institute, MA). The IL-6 human probe cloned was purchased from the American Type Collection (Rockville, MD, USA), and human cyclophilin A probe was provided by Dr. FX Real (IMIM, Barcelona, Spain). Densitometry was performed using a Gel Doc 2000 BioRad (Bio-Rad Laboratories, Hercules, CA) scanning densitometer, and densitometry curve integration and analysis was accomplished using the Molecular Analysis software package (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Data are expressed as mean ± SEM. Hierarchically adjusted analysis of variance (ANOVA) was used to compare the means of different quantitative variables among groups with inde-

pendent data. When ANOVA detected statistically significant differences, concentrations tested in each experiment were compared using the Scheffe method. A $P \leq 0.05$ was considered statistically significant. Analysis was performed using the SPSS statistical package (6.1.3, Copyright (c) SPSS Inc., 1989–1995).

Results

Effects of Alendronate and Etidronate on Stimulated IL-6 Production in Normal Human Osteoblasts

The results of our study show that normal human osteoblasts constitutively produce IL-6 (Fig. 2). The detected concentration in culture supernatants was 2.490 ± 106 pg/30,000 cells. A statistically significant increase was observed ($P = 0.0005$) in IL-6 concentration when cultures were treated with TNF-α, IL-1β, or TNF-α + IL-1β. These increases were higher when the culture medium was supplemented with FCS. In addition, significant differences ($P = 0.0005$) were also detected in the degree of stimulation achieved with TNF-α, IL-β, or TNF-α + IL-1β. The highest degree of stimulation in IL-6 production was achieved with the combination TNF-α + IL 1β, followed by IL-1β, and by TNF-α (Fig. 1).

In our study, no significant changes in IL-6 concentration were detected in supernatants of cultures treated with different alendronate or etidronate concentrations. These results were obtained in both nonstimulated cultures and in cultures stimulated by means of TNFα, IL-1β, and TNFα + IL-1β, with or without FCS (Fig. 2). IL-6 was not detected when the culture medium was analyzed with and without FCS.

Effects of Alendronate and Etidronate on Stimulated IL-11 Production in Normal Human Osteoblasts

On analyzing IL-11 concentration in the supernatant of nonstimulated human normal osteoblast cultures, constitutive production of this cytokine was not found (Fig. 4). IL-11values, when the cultures were stimulated with TNF-α without the addition of FCS, were below the detection limit of our technique (8 pg/ml). When FCS was added to cultures treated with TNF-α, detectable levels of IL-11 were found in the supernatants. Stimulation with IL-1β or with a combination of TNF-α + IL-1β was also associated with detectable IL-11 levels, regardless of whether FCS was added to the medium. Statistically significant differences ($P = 0.0005$) were found between stimulation achieved with IL-1β and TNFα + IL-1β. The highest level of stimulation of IL-11 production was obtained with the combination TNFα + IL1β, with the addition of FCS (Fig. 3).

In the present study, no significant changes were found in supernatant concentration of IL-11 among

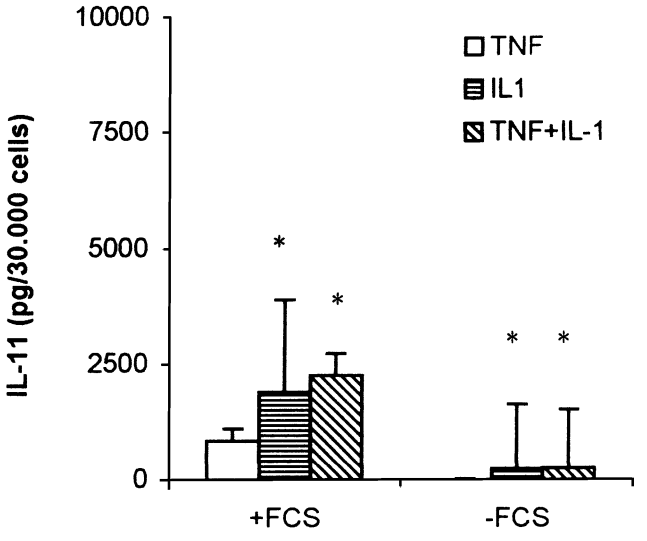

Fig. 3. Effect of TNF-α (0.3 nm) and IL-1β (0.2 nm) on IL-11 induction in human osteoblasts. Cultures were treated for 24 h in the presence or absence of TNF-α, IL-1β, or TNF-α + IL-1β, with or without FCS. The conditioned media were assessed for IL-11 production by a human IL-11-specific ELISA. IL-11 synthesis in cultures treated with IL-1β or TNF-α + IL-1β showed a statistically significant increase compared with those treated with TNF-α alone, with and without FCS. In the absence of FCS, levels of IL-11 in cultures treated with TNF-α were not detectable. Data are expressed as mean ± SEM (n = 3, $*P \leq 0.0005$)

cultures treated with six different alendronate (10^{-7} and 10^{-12} M) or etidronate (10^{-4} and 10^{-9} M) concentrations. These results were obtained both in nonstimulated cultures and in those stimulated with TNFα, IL-1β, and TNFα + IL-1β, either with or without FCS (Fig. 4). IL-11 was not detected when the culture medium was analyzed with and without FCS.

Effects of Alendronate and Etidronate on mRNA IL-6 and mRNA IL-11 Levels

In lysates of osteoblast cultures, constitutive presence of IL-6-codifying but not of IL-11-codifying mRNA was detected with Northern blot. Weak IL-11 expression could be detected when FCS was added to the cultures. After stimulation of cultures with TNF-α, IL-1β, or TNF-α + IL1β, both IL-6 and IL-11-codifying mRNA levels increased. The highest levels were obtained with the combination TNF-α + IL1β and FCS. Their sizes (~2.5 and 1.5 kilobases) (Fig. 5A and 5B) are in accord with the IL-11 mRNA transcripts described by Elias et al. [25] in human lung fibroblasts and epithelial-like cells.

In cultures treated with three different alendronate (10^{-7}, 10^{-9}, 10^{-12} M) and etidronate (10^{-4}, 10^{-6}, 10^{-9} M) concentrations and stimulated with TNF-α + IL1β and FCS, no differences were detected in levels

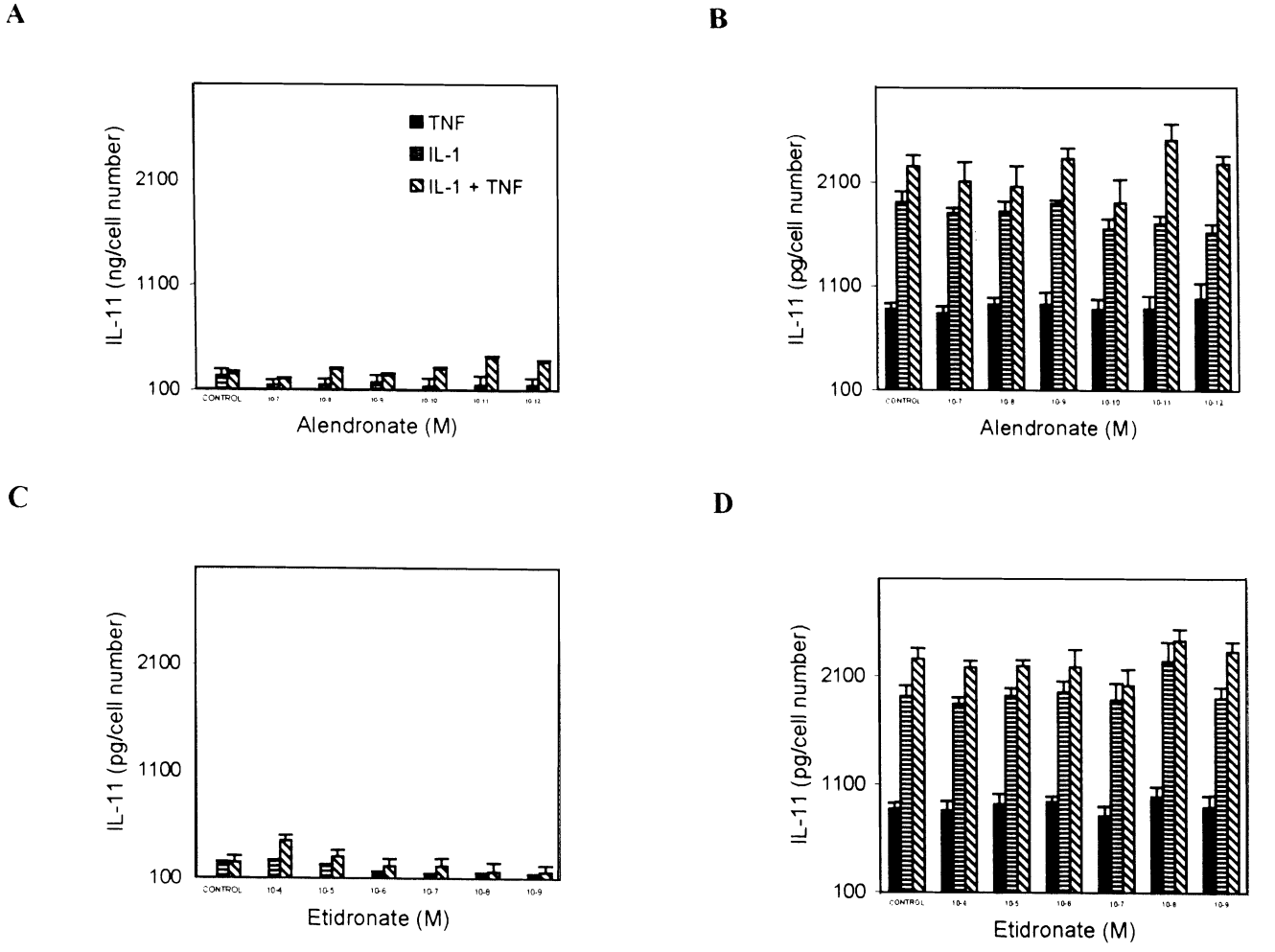

Fig. 4. Effect of different doses of alendronate and etidronate on TNF-α, IL-1β, or TNF-α + IL-1β-induced IL-11 secretion in human osteoblasts. Cultures were treated for 24 h with alendronate or etidronate in the presence or absence of TNF-α, IL-1β, or TNF-α + IL-1β, with or without FCS. **(A)** Cultures treated with alendronate without FCS, **(B)** alendronate with FCS, **(C)** etidronate without FCS, **(D)** etidronate with FCS. The conditioned media were assessed for IL-11 production by a human IL-11-specific ELISA. No effect of any of the alendronate or etidronate concentrations tested was observed on IL-11 synthesis in the cultures, with or without TNF-α, IL-1β, or TNF-α + IL-1β stimulation, with or without FCS. Data are expressed as mean ± SEM (n = 3)

of IL-6-codifying or IL-11-codifying mRNA (Fig. 5A and 5B).

Discussion

Our results show that non-neoplastic human osteoblasts secrete IL-6 constitutively *in vitro*. Other authors previously detected constitutive synthesis of IL-6 in a cell line obtained from human (MG63) or murine (UMR-106) osteosarcoma [28, 29] and in non-neoplastic human osteoblasts [30]. Our study confirms that stimulation of non-neoplastic human osteoblasts with IL-1β and TNF-α intensely increases IL-6 synthesis [30]. Of both stimulating factors, IL-1β is the most powerful. The combined addition of IL-1β and TNF-α resulted in the highest increase in IL-6 secretion. As expected, the increase in cytokine production was greater when FCS was added to the culture medium than when it was not. The present study provides the first demonstration that human osteoblasts do not have constitutive secretion of IL-11 *in vitro*. Should this secretion actually take place, it should be well below the sensitivity level of our ELISA assay, i.e., 8 pg/ml. In this regard, we did not detect constitutive expression of IL-11 mRNA by Northern blot. In addition, our results show for the first time that IL-1β is a much stronger stimulator of osteoblastic IL-11 secretion than TNF-α, since the latter is only effective when FCS is added to the culture medium. The addition of both IL-1β and TNF-α resulted in the maximum increase in secreted IL-11 levels. Accordingly, our findings are consistent with the hypothesis that, in physiological conditions, the modulating role of IL-6 and IL-11 in

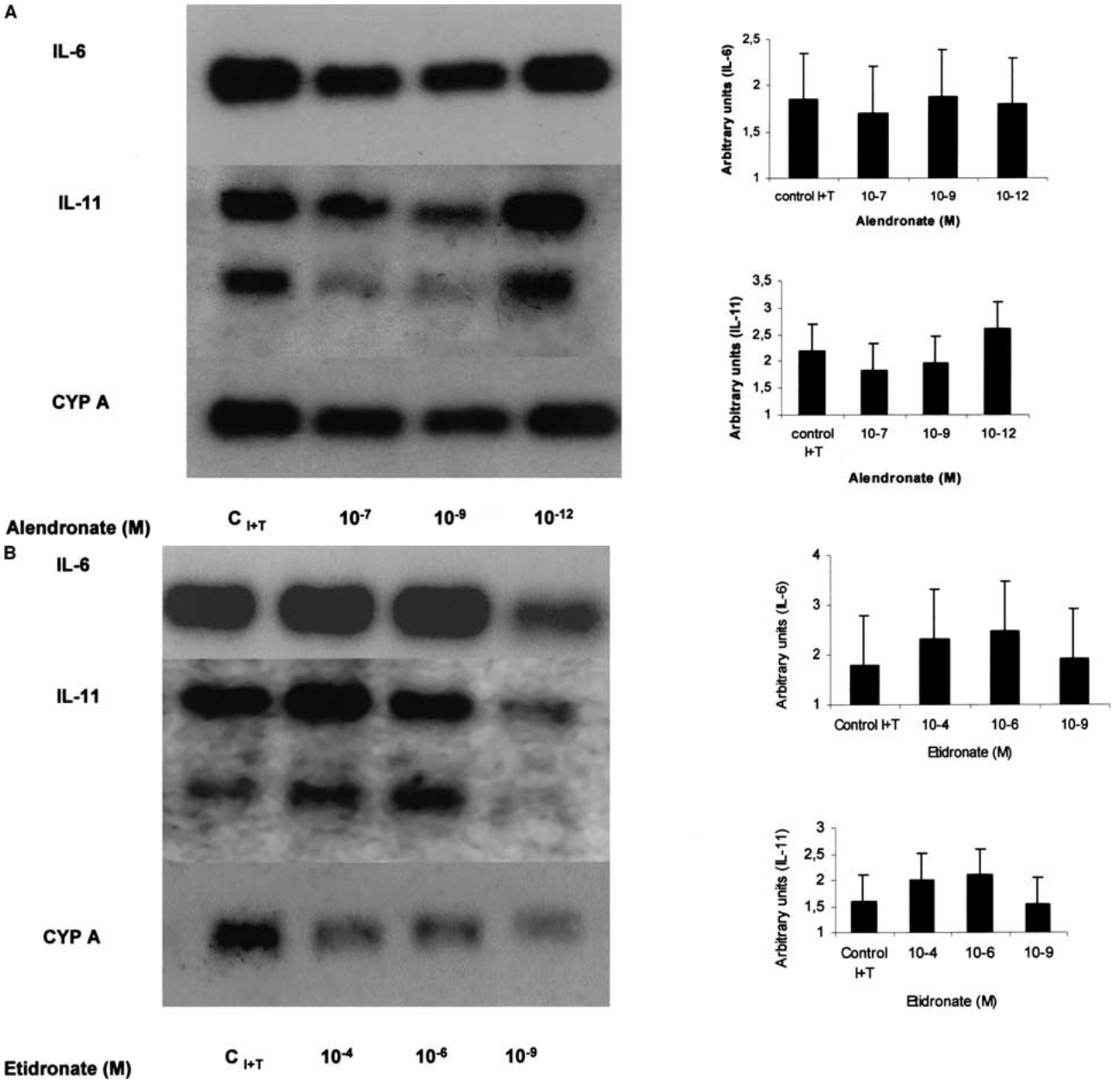

Fig. 5. Northern blot analysis of IL-6 and IL-11 after treatment of human osteoblasts with alendronate or etidronate. Cultures were treated with TNF-α + IL-1β in the presence of FCS. Three concentrations of **(A)** alendronate (10^{-7}, 10^{-9}, 10^{-12} M) or **(B)** etidronate (10^{-4}, 10^{-6}, 10^{-9} M) were tested. Total RNA was isolated and subjected to Northern blot analysis. Neither alendronate nor etidronate affected IL-6 or IL-11 mRNA levels. Cyclophilin A mRNA expression was determined as the internal control. (Mean ± SEM of three independent experiments. $P < 0.05$ Scheffe's *f*-test)

bone resorption may be mediated, at least in part, by IL-1β and TNF-α [18].

In the present study, we did not observe that alendronate and etidronate had any effect on osteoblastic IL-6 and IL-11 secretion or on the expression of their respective mRNAs. This lack of effect was confirmed for all tested concentrations of alendronate and etidronate stimulated with IL-1β, TNF-α, or the combination of both. This was also the case when cultures were not stimulated, permitting us to rule out the possibility that these results were due to excessive cell stimulation. On the other hand, in a previous study, we showed that concentrations of alendronate and etidronate used in the present experiment did not affect viability, proliferation, or mineral deposit capacity of non-tumoral human osteoblasts in culture [31].

A previous study by Giuliani et al. [28] showed that bisphosphonates inhibited the production of IL-6 induced by IL-1β and TNF-α in MG-63 osteoblast-like cells obtained from a human osteosarcoma. The dis-

crepancy between these results and those of our study are probably derived from differences in the experimental models used. First of all, every experiment in the present study was performed on homogeneous lines of non-neoplastic human osteoblasts obtained from primary cultures of cells showing a mature osteoblast phenotype. This system is much more representative of normal physiology than osteogenic sarcoma cell lines, in which cytokine production might be altered by transformation-related changes [30]. On the other hand, Giuliani et al. assessed the effects of bisphosphonates on IL-6 secretion by means of a bioassay. It is well known that this is an indirect method, as it does not quantify the secreted protein but rather measures its activity. Instead of this, the present study was performed using sensitive quantitative ELISA techniques, permitting concentrations of specific cytokines in the conditioned medium to be quantified. Moreover, results of ELISAs concurred with those of Northern blot analysis of IL-6 mRNA levels.

In contrast, Sanders et al. [29] studied the effect of alendronate on IL-6 production by two different models: UMR-106 rat osteoblastic osteosarcoma cells and fetal rat limb bone culture. The results of their study contradicted those of Giuliani et al. Thus, Sanders et al. obtained an increase in IL-6 secretion when treating the cultures with alendronate. However, this was only observed under intensive stimulation with IL-1β. Interestingly, in the fetal rat limb bone culture model, dissociation was observed between the effect of alendronate on resorption and that on IL-6 levels. Again, it is conceivable that the differences between our results and those of Sanders et al. are due to differences in culture models and in the methods used for quantification. Once more, the model used in the present study is more representative of normal physiology than those using rat osteosarcoma cell lines or fetal and neonatal rat cells. It is well known that transformed and fetal cells have very different biological behavior compared with human non-neoplastic adult cells. Furthermore, in bone organ culture, cytokine production by marrow cells other than osteoblasts may be a confounding factor. Finally, Sanders et al., like Giuliani et al., used a bioassay as a quantification method, with the limitations we mentioned before.

Although it has been shown that IL-6 and IL-11 are interleukins that play an important role in the activation of bone resorption [23, 32], the results of our study allow us to conclude that alendronate and etidronate do not operate indirectly on resorption by modifying these cytokine levels. In any event, it is not possible to rule out the possibility that an effect of bisphosphonates on osteoblast-mediated resorption could occur. In this way, knowledge of osteoclast activity inhibitors [33–35] has led some authors to suggest that indirect action of bisphosphonates on resorption would not be exerted through the inhibition of resorption-stimulating osteoblastic factor secretion, but rather by stimulating the secretion of resorption-inhibiting factors. In this regard, Nishikawa et al. [36] and Vitté et al. [13] demonstrated that bisphosphonates stimulate secretion of osteoclast-inhibiting molecules by osteoblasts.

In conclusion, the present study shows that human non-neoplastic osteoblasts have no native synthesis of IL-11 *in vitro*. In addition, this study also demonstrates that alendronate and etidronate do not regulate the synthesis of IL-6 and IL-11 in these cells. Therefore, a possible indirect, osteoblast-mediated action of bisphosphonates on bone resorption would not be exerted through reduction in osteoblastic synthesis of IL-6 and IL-11.

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